Amendments to the Specification

Please replace the paragraph at page 1, lines 2 through 7 with the following amended paragraph:

~~**Background of Invention**~~

RELATED APPLICATIONS

This application is a continuation application of U.S. Application No. 09/603,713, filed June 27, 2000, which claims priority to U.S.S.N. 60/141,363 filed June 28, 1999 by Lin, et al., U.S.S.N. 60/168,060 filed November 30, 1999 by Lin, et al., U.S.S.N. 60/177,836 filed January 25, 2000 by Lin, et al., U.S.S.N. 60/178,368 filed January 27, 2000 by Lin, et al., and U.S.S.N. 60/210,292 filed June 8, 2000 by Lin Hong, et al., the teachings of all of which are hereby incorporated by reference ~~therein~~ in their entirety.

BACKGROUND OF THE INVENTION

Please replace the paragraph at page 7, lines 12 through 20 with the following amended paragraph:

Figure 1 depicts the plasmid construct of vector pET-11a-memapsin 2-T1 and pET-11a-memapsin 2-T2. The T7 promotor, amino acid sequence from the vector (T7 protein) (SEQ ID NO:3), and the beginning and ending of the memapsin 2 T1 and T2 construct are shown. Construct promemapsin 2-T1 was used in the preparation of protein for crystallization and includes residues 1 v-15v which are derived from vector pET-11a. Residues 1p-48p are putative propeptide. Residues 1-393 correspond to the mature protease domain and C-terminal extension. The residue numbering of memapsin 2 starts at the aligned N-terminal position of pepsin (~~Figure 3~~ Figures 3A and 3B).

Please replace the paragraph at page 7, lines 25 through 26 with the following amended paragraph:

Figures 3A and 3B are the chemical structures of memapsin 2 inhibitors, ~~OM99-2 and OM99-1~~ OM99-1 and OM99-2.

Please replace the paragraph at page 8, lines 1 through 6 with the following amended paragraph:

Figure 6 is a stereo view of crystal structure of memapsin 2 protease domain with bound OM99-2.The polypeptide backbone of memapsin 2 is shown as a ribbon diagram. The N-lobe and C- lobe are blue and yellow, respectively, except the insertion loops (designated A to G, see ~~Figure 2~~ Figure 6) in the C-lobe are magenta and the C-terminal extension is green. The inhibitor bound between the lobes is shown in red.

Please replace the paragraph at page 19, lines 5 through 12 with the following paragraph:

(1) The primary specificity site for a memapsin 2 substrate is [subsite] position, P_1'. This means that the most important determinant for substrate specificity in memapsin 2 is the amino acid[, S1'] at P_1'. P_1' must [contain] be a small side chain for memapsin 2 to recognize the substrate. Preferred embodiments are substrate analogs where R_1 of the P_1' position is either H (side chain of glycine), CH_3 (side chain of alanine), CH_2OH (side chain of serine), or [CH_2OOH] CH_2COOH (side chain of aspartic acid). Embodiments that have an R1 structurally smaller than CH_3 (side chain of alanine) or CH_2OH (side chain of serine) are also preferred.

Please replace the paragraph at page 44, lines 13 through 18 with the following amended paragraph:

Enzyme activity was measured as described above, but with the addition of either OM99-1 or OM99-2. OM99-1 inhibited recombinant memapsin 2 as shown in ~~Figure 5A~~ Figure 4A. The Ki calculated is 3×10^{-8} M. The substrate used was a synthetic fluorogenic peptide substrate. The inhibition of OM99-2 on recombinant memapsin 2 was measured using the same fluorogenic substrate. The Ki value was determined to be 9.58×10^{-9} M, as shown in ~~Figure 5B~~ Figure 4B.

Please replace the paragraph at page 46, lines 12 through 23 with the following paragraph:

This memapsin 2 solution was allowed to stand at 4 °C for 2-3 weeks. The total volume of approximately 16 liters was concentrated to 40 mls using ultra-filtration (Millipore) and stir-

cells (Amicon), and centrifuged at 140,000 xg at 30 minutes in a rotor [pre-equilbrated] pre-equilibrated to 4 °C. The recovered supernatant was applied to a 2.5 x 100 cm column of S-300 equilibrated in 0.4 M urea, 20 mM Tris-HCl, pH 8.0, and eluted with the same buffer at 30 ml/hour. The active fraction of memapsin 2 was pooled and further purified in FPLC using a 1 ml [Resource-Q] Resource-Q® (Pharmacia Biotech 1997, page 195) column. Sample was filtered, and applied to the [Resource-Q] Resource-Q® column equilibrated in 0.4 M urea, 50 mM Tris-HCl, pH 8.0. Sample was eluted with a gradient of 0 - 1 M NaCl in the same buffer, over 30 ml at 2 ml/min. The eluents containing memapsin 2 appeared near 0.4 M NaCl which was pooled for crystallization procedure at a concentration near 5 mg/ml.

Please replace the paragraph at page 46, line 28 through page 47, line 3 with the following paragraph:

The activation of the folded pro-enzyme to mature enzyme, memapsin 2, was carried out as described above, i.e., incubation in 0.1 M sodium acetate pH 4.0 for 16 hours at 22 °C. Activated enzyme was further purified using anion-exchange column chromatography on [Resource-Q] Resource-Q® anion exchange column. The purity of the enzyme was demonstrated by SDS-gel electrophoresis. At each step of the purification, the specific activity of the enzyme was assayed as described above to ensure the activity of the enzyme.

Please replace the paragraph at page 47, line 22 through page 48, line 3 with the following amended paragraph:

Crystals of memapsin-inhibitor complex were obtained at 30% PEG 8000, 0.1 M NaCocadylate, pH 6.4. SDS gel electrophoresis of a dissolved crystal verified that the content of the crystal to be memapsin 2. Several single crystals (with the sizes about 0.3 min x 0.2 mm x 0.1 mm) were carefully removed from the cluster for data collection on a Raxis IV image plate. These results showed that the crystals diffract to 2.6 Å. ~~A typical protein diffraction pattern is shown in Figure 6.~~ An X-ray image visualization and integration software-Denzo, was used to visualize and index the diffraction data. Denzo identified that the primitive orthorhombic lattice has the highest symmetry with a

significantly low distortion index. The unit cell parameters were determined as: a=89.1 Å, b=96.6 Å, c=134.1 Å, $\alpha=\beta=\gamma=90^{o}$. There are two memapsin 2/OM99-2 complexes per crystallographic asymmetric unit, the V_m of the crystal is 2.9 $Å^3$/Da. Diffraction extinctions suggested that the space group is $P2_12_12_1$.

Please replace page 51, lines 1 through 30 with the following:

Table 2. Data Collection and Refinement Statistics

A. Data Statistics	
Space group	$P2_1$
Unit Cell (a, b, and c in Å)	53.7, 85.9, 109.2
(α, β, and γ in degrees)	90.0, 101.4, 90.0
Resolution (Å)	25.0-1.9
Number of observed reflections	144,164
Number of unique reflections	69,056
R_{merge} [a]	0.061 (0.25)
Data completeness (%) (25.0-1.9)	90.0 (68.5)
<I/ σ (I)>	13.7 (3.0)
B. Refinement Statistics	0.186
$R_{working}$ [b]	0.186
R_{free} [b]	0.228
RMS deviation from ideal values	
Bond length (Å)	0.014
Bond angle (DEG)	1.7
Number of water molecules	445
Average B-factor ($Å^2$)	
Protein	28.5
Solvent	32.2

[a] $R_{merge} = \Sigma_{hkl} \Sigma_i | I_{hkl,i} - \langle I_{hkl} \rangle | / \Sigma_{hkl} \langle I_{hkl} \rangle$, where $I_{hkl,i}$ is the intensity of the ith measurement and $\langle I_{hkl} \rangle$ is the weighted mean of all measurements of I_{hkl}. [b] $R_{working\ (free)} = \Sigma | | F_o | - | F_c | | / \Sigma | F_o |$, where F_o and F_c are the observed and calculated structure factors. Number in parentheses are the corresponding numbers for the highest resolution shell (2.00-1.9 Å). Reflections with $F_o / \sigma (F_o) >= 0.0$ are included in the refinement and R factor calculation.

Please replace the paragraph at page 52, lines 2 through 13 with the following amended paragraph:

The bilobal structure of memapsin 2 (~~Figure 7~~ Figure 6) is characteristic of aspartic proteases (Tang, J., et al., Nature 271, 618-621 (1978)) with the conserved folding of the globular core. The substrate binding cleft, where the inhibitor is bound (~~Figure 7~~ Figure 6), is located between the two lobes. A pseudo two-fold symmetry between the N- (residues 1-180) and C- (residues 181-385) lobes (~~Figure 7~~ Figure 6), which share 61 superimposable atoms with an overall 2.3 Å rms deviation using a 4 Å cutoff. The corresponding numbers for pepsin are 67 atoms and 2.2 Å. Active-site Asp^{32} and Asp^{228} and the surrounding hydrogenbond network are located in the center of the cleft (~~Figure 7~~ Figure 6) and are conserved with the typical active-site conformation (Davies, D. R., Annu. Rev. Biophys. Chem. 19, 189 (1990)). The active site carboxyls are, however, not co-planar and the degree of which (50°) exceeds those observed previously.

Please replace the paragraph at page 52, line 14 through page 53, line 6 with the following amended paragraph:

Compared to pepsin, the conformation of the N-lobe is essentially conserved (Sielecki et al., 1990). The most significant structural differences are the insertions and a C-terminal extension in the C-lobe. Four insertions in helices and loops (~~Figure 7~~ Figure 6) are located on the adjacent molecular surface. Insertion F, which contains four acidic

residues, is the most negatively charged surface on the molecule. Together, these insertions enlarged significantly the molecular boundary of menapsin 2 as compared to pepsin (~~Figure 8~~ Figure 7). These surface structural changes may have function in the association of memapsin 2 with other cell surface components. Insertions B and E are located on the other side of the molecule (~~Figure 7~~ Figure 6). The latter contains a beta-strand that paired with part of the C-terminal extension G. A six- residue deletion occurs at position 329 on a loop facing the flap on the opposite side of the active-site cleft, resulting in an apparently more accessible cleft. Most of the C-terminal extension (residues 359-393) is in highly ordered structure. Residues 369-376 form a beta structure with 7 hydrogen bonds to strand 293-299, while residues 378-383 form a helix (~~Figures 7 and 8~~ Figures 6 and 7). Two disulfide pairs (residues 155/359 and 217/382) unique to memapsin 2 fasten both ends of the extension region to the C-lobe. This C-terminal extension is much longer than those observed previously and is conformationally different [Cutfield, S. M., et al., Structure 3, 1261 (1995); Abad-Zapatero, C., et al., Protein Sei. 5, 640 (1996); Symersky, J. et al., Biochemistry 36, 12700 (1997); Yang, J., et al., Acta Crystallogr. D 55, 625 (1999)]. The last eight residues (386-393) are not seen in the electron density map; they may form a connecting stem between the globular catalytic domain and the membrane anchoring domain.

Please replace the paragraph at page 53, lines 16 through 30 with the following amended paragraph:

The binding of the eight-residue inhibitor OM99-2 in the active-site cleft shares some structural features with other aspartic protease-inhibitor complexes [Davies, D.R., Annu. Rev. Biophys. Chem. 19, 189 (1990); Bailey and Cooper, (1994); Dealwis et al., (1994)]. These include four hydrogen bonds between the two active-site aspartics to the hydroxyl of the transition-state isostere, the covering of the flap (residues 69-75) over the central part of the inhibitor and ten hydrogen bonds to inhibitor backbone (~~Figure 9~~ Figures 8 and 9). Most of the latter are highly conserved among aspartic proteases [Davies, D. R. Annu. Rev. Biophys. Chem. 19, 189 (1990); Bailey and Cooper, (1994); Dealwis et al., (1994)] except that hydrogen bonds to Gly^{11} and Tyr^{198} are unique to

memapsin 2. These observations illustrate that the manner by which mernapsin 2 transition-state template for substrate peptide backbone and mechanism. of catalysis are similar to other aspartic proteases. These common features are, however, not the decisive factors in the design of specific memapsin 2 , nhibitors with high selectivity.

Please replace the paragraphs at page 54, lines 1 through 30 with the following amended paragraph:

The observation important for the design of inhibitor drugs is that the memapsin 2 residues in contact with individual inhibitor side chains (~~Figure 9~~ Figure 8) are quite different from those for other aspartic proteases. These side chain contacts are important for the design of tight binding inhibitor with high selectivity. Five N-terminal residues of OM99-2 are in extended conformation and, with the exception of P_1' Ala, all have clearly defined contacts (within 4 Å of an inhibitor side chain) with enzyme residues in the active-site cleft (~~Figure 9~~ Figure 8).

The protease S_4 subsite is mostly hydrophilic and open to solvent. The position of inhibitor P_4 Glu side chain is defined by hydrogen bonds to Gly^{11} and to P_2 Asn (~~Figure 9~~ Figure 8) and the nearby sidechains of Arg^{235} and Arg^{307}, which explains why the absence of this residue from OM99-2 cause a 10-fold increase in K_i. Likewise, the protease S_2 subsite is relatively hydrophilic and open to solvent. Inhibitor P_2 Asn side chain has hydrogen bonds to P_4 Glu and Arg^{235}. The relatively small S_2 residues Ser^{325} and Ser^{327} (Gln and Met respectively in pepsin) may fit a side chain larger than Asn. Memapsin 2 S_1 and S_3 subsites, which consist mostly of hydrophobic residues, have conformations very different from pepsin due to the deletion of pepsin helix h_{H2} (Dealwis, et al., (1994)). The inhibitor side chains of P_3 Val and P_1 Leu are closely packed against each other and have substantial hydrophobic contacts with the enzyme (~~Figure 9~~ Figure 8), especially P_3 interacts with Tyr^{71} and Phe^{108}. In the beta- secretase site of native APP, the P_2 and P_1 residues are Lys and Met respectively. Swedish mutant APP has Asn and Leu in these positions respectively, resulting in a 60-fold increase of k_{cat}/K_m, over that for native APP and an early onset of AD described by Mullan, M., et al. [Nat. Genet. 2, 340 (1992)]. The current structure suggests that inhibitor P_2 Lys would place its positively charge in an

unfavorable interaction with Arg^{235} with a loss of hydrogen bond to Arg^{235} while P_1 Met would have less favorable contact with memapsin 2 than does leucine in this site (~~Figure 10~~ Figure 9). No close contact with memapsin 2 was seen for P_1' Ala and an aspartic at this position, as in APP, may be accommodated by interacting with Arg^{228}.

Please replace the paragraph at page 55 , lines 1 through 13 with the following amended paragraph:

The direction of inhibitor chain turns at P_2' and leads P_3' and P_4' toward the protein surface (~~Figure 10~~ Figure 9). As a result, the side-chain position of P_2' Ala deviates from the regular extended conformation. The side chains of P_3' Glu and P_4' Phe are both pointed toward molecular surface with little significant interaction with the protease (~~Figure 10~~ Figure 9). The relatively high B-factors (58.2 $Å^2$ for Glu and 75.6 $Å^2$ for Phe) and less well-defined electron density suggests that these two residues are relatively mobile, in contrast to the defined structure of the S_3' and S_4' subsites in renin-inhibitor (CH-66) complex (Dealwis et al., 1994). The topologically equivalent region of these renin subsites (residues 292 - 297 in pepsin numbering) is deleted in memapsin 2. These observations suggest that the conformation of three C-terminal residues of OM99-2 may be a functional feature of memapsin 2, possibly a way to lead a long protein substrate out of the active-site cleft.

Please replace the paragraph at page 55, lines 15 through page 56, line 2 with the following amended paragraph:

Pharmaceutically acceptable inhibitor drugs normally post a size limit under 800 daltons. In the case of memapsin 2 inhibitors, this requirement may even be more stringent due to the need for the drugs to penetrate the blood-brain barrier [Kearney and Aweeka, (1999)]. In the current model, well defined subsite structures spending P_4 to P_2' provide sufficient template areas for rational design of such drugs. The spacial relationships of individual inhibitor side chain with the corresponding subsite of the enzyme as revealed in this crystal structure permits the design of new inhibitor structures in each of these positions. It is also possible to incorporate the unique conformation of

subsites P_2', P_3' and P_4' into the selectivity of memapsin 2 inhibitors. The examples of inhibitor design based on the current crystal structure are given below.

Example A: Since the side chains of P_3 Val and P_1 Leu are packed against each other and there is no enzyme structure between them, cross-linking these side chains would increase the binding strength of inhibitor to memaspin 2. This is because when binding to the enzyme, the cross-linked inhibitors would have less entropy difference between the free and bound forms than their noncross-linked counterparts [Khan, A.R., et al., Biochemistry, 37, 16839 (1998)]. Possible structures of the cross-linked side chains include those shown in ~~Figure 11~~ Figure 10.

Please replace the paragraph at page 56, lines 3 through 14 with the following amended paragraph:

Example B: The same situation exits between the P4 Glu and P2 Asn. The current crystal structure shows that these side chains are already hydrogen bonded to each other so the cross linking between them would also derive binding benefit as described in the Example A. The cross-linked structures include those shown in ~~Figure 12~~ Figure 11.

Example C: Based on the current crystal structure, the P1' Ala side chain may be extended to add new hydrophobic, Van der Waals and H-bond interactions. An example of such a design is diagramed in ~~Figure 13~~ Figure 12.

Example D: Based on the current crystal structure, the polypeptide backbone in the region of P1, P2, and P3, and the side chain of P1-Leu can be bridged into rings by the addition of two atoms (A and B in ~~Figure 14~~ Figure 13). Also, a methyl group can be added to the beta-carbon of the P1-Leu (~~Figure 14~~ Figure 13).